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Hydrolase Activities of Sourdough Microorganisms

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Abstract: Sourdough is renowned for improving bakery products' nutritional and quality characteristics through the enzymes produced by its microbiota. Among the enzymatic framework present in sourdough fermentation, amylase, cellulase, and peptidase are responsible for many of the properties valued in sourdough products. Furthermore, there is an increasing concern regarding the allergenic potential of gluten, which motivates the investigation of enzymatic gluten hydrolysis. This study aimed to select probiotics, isolate and identify microorganisms from sourdough, and assess their amylase, cellulase, and peptidase profiles. Additionally, a rapid screening method was developed for gluten and wheat flour hydrolysis, and gluten zymography and enzymography were performed. As a result, 18 microorganisms were isolated from sourdough and identified. The probiotic *Bacillus licheniformis* LMG-S 28935, and three microorganisms isolated from sourdough, the *Limosilactobacillus fermentum*, *Pediococcus pentosaceus*, and *Saccharomyces cerevisiae*, completed the profile of analyzed hydrolases and presented the capacity to hydrolyze gluten. These findings contribute to a better understanding of sourdough microorganisms' hydrolase activities in the bakery science and technology field. In addition, an efficient, fast, and economical method for screening extracellular glutenase, produced by microorganisms, was applied. To our knowledge, it was the first time that amylase, cellulase, and peptidase activities were assessed from sourdough microorganisms.

Keywords: *Lactobacillus*; gluten; hydrolases; microorganisms; probiotics; sourdough; yeasts

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1. Introduction

Natural sourdough fermentation occurs due to microbiota activity in the fermented flour, mainly due to *Lactobacillus* and yeasts. These microorganisms are related to baked products' sensory and nutritional quality, and one important function is enzyme production. There are three enzymes sources in sourdough: the endogenous enzymes in flour used, enzymes from microorganisms during the fermentation process, and exogenous enzymes, which can be added to the dough [1]. This study focused on extracellular hydrolases belonging to peptidases, amylases, and cellulase classes secreted by microorganisms used as starters in sourdough.

Peptidases act in proteins present in the dough. Wheat gluten proteins account for 80% of the total wheat protein and are major determinants of its baking quality. Depending on several factors, gluten protein is associated with wheat-related disorders, and its adverse effects can cause health problems, from allergic reactions to celiac disease [2].

Gluten comprises proteins found in grains such as wheat, barley, and rye. The two main proteins of gluten are gliadin and glutenin. Gliadins are monomeric proteins and comprise the types α/β (28,000–35,000 MW), γ-gliadins (31,000–35,000 MW), ω1,2 (39,000–44,000 MW), and ω5 (49,000–55,000 MW). It is an alcohol-soluble protein responsible for most of the adverse effects of gluten on people with gluten sensitivity or intolerance. Glutenin is water- and ethanol-insoluble but soluble in dilute acids. It is a polymeric protein linked by interchain disulfide (SS) bonds with a wide MW distribution ranging from 105 to several million. Glutenin, for example, presents a varying size ranging from about 500,000

to more than 10 million [3]. Glutenin subunits exist in two types: high-molecular-weight (HMW–GS) (70,000–90,000 MW) and LMW–GS (20,000–45,000 MW) [3–5]. HMW-GS is responsible for wheat flour dough's elasticity. Gliadins and LMW-GS are responsible for the viscous properties [6]. The proportion of gluten proteins varies considerably depending on genetic and environmental factors [7,8].

Gluten degradation by peptidases during sourdough fermentation is a crucial phenomenon affecting sourdough bread quality. Besides this, immunogenicity reduction is linked to decreasing gluten, explicitly weakening the polymerization ability of glutenin and hydrolyzing glutenin peptides [9]. The amino acids and peptides produced by proteolysis affect the taste of fermented foods and, in particular, are essential precursors for volatile flavor compounds [10]. In addition, it has been described that a limited amount of proteolysis during sourdough fermentation beneficially improves the bread flavor without adversely affecting texture and volume [6]. Other wheat proteins are the water/salt-soluble albumins and globulins known to cause typical IgE-mediated wheat allergy [11]. In bread wheat, a type of globulin, the α-amylase/trypsin inhibitors (ATI) represent up to 4% of total wheat proteins and consist of at least 14 types of subunit protein. Recently, ATI has been identified as an inducer of an innate immune response via toll-like receptor 4 in celiac disease and non-celiac wheat sensitivity. This protein is resistant primarily to gastrointestinal proteases and heat, and their inflammatory activity affects the intestine and peripheral organs. A recent study demonstrated that sourdough fermentation degraded ATI structure and bioactivity [12]. The study of peptidases involved in sourdough fermentation leads to a better understanding of these mechanisms and points to new possibilities for reducing the allergenicity of wheat proteins while maintaining a higher organoleptic, nutritional quality of wheat dough. Investigations revealed that a low pH environment, microbial proteolytic system, and wheat enzymes involved in sourdough enabled the allergenicity and toxicity reduction in wheat proteins during fermentation [9,13].

Another hydrolase present in sourdough is amylase. Amylase can come from the flour and the microorganisms in the sourdough, and specifically targets and hydrolyses alpha-1,4-glycosidic bonds within starch molecules, converting them into maltose and other smaller sugar units, providing a nutrient source for the yeast and lactic acid bacteria [14]. Yeast consumes the sugars from amylase, converting them into carbon dioxide and alcohol through fermentation. This carbon dioxide creates air pockets within the dough, leading to its rise during proofing and baking [15]. Lactic acid bacteria consume the sugars and produce lactic acid as a byproduct. The lactic acid contributes to the sour flavor. It helps create an acidic environment that inhibits the growth of harmful bacteria [16]. The enzyme improver helps to increase the loaf volume, lowers the crumb firmness, and keeps bread fresh for longer [14].

Cellulases are not typically studied in the microorganisms present in sourdough. However, the addition of the enzyme in sourdoughs has been studied. In Chinese steamed bread dough enriched in wheat bran, cellulase addition significantly increased the development time, stability, departure time, mixing tolerance index (MTI), extensibility, and stickiness of the regular dough. It decreased both softening and resistance to extension [17]. A study by Liu et al. [18] demonstrated that by combining α-amylase, xylanase, and cellulase, a better rheological effect is obtained with the synergism.

Enzymes in sourdough fermentation is a broad field of research, and still needs many studies, considering the multiplicity of factors involved and the complexity of the microbiota. It is interesting to work with co-cultures aiming to improve health properties and quality of bread; for instance, a co-culture of *Pediococcus acidilactici* and yeast improved the digestibility of wheat protein compared with single-strain fermentation [11].

The present study points out that the microbial enzymes from native sourdough microorganisms could be a strategy to improve product development for better bread with excellent properties, decreasing wheat sensitivity in the susceptible population.

2. Materials and Methods

2.1. Materials

The Brain Heart Infusion media (BHI) was obtained from Neogen. De Man, Rogosa e Sharp medium (MRS), and yeast extract were provided by Kasvi (São José dos Pinhais, PR, Brazil), and peptone was obtained from Himedia (Kennett Square, PA, USA). Soluble starch was purchased from Reagen (Colombo, PR, Brazil), gelatin from Vetec (Duque de Caxias, RJ, Brazil), and lugol solution from Laborclin (Pinhais, PR, Brazil). Other reagents were carboxymethylcellulose, Coomassie brilliant blue R-250, and dithiothreitol (DTT) (Sigma-Aldrich, Burlington, MA, USA). SDS-PAGE standards broad range was provided by Bio-Rad (Hercules, CA, USA). The flours used were white wheat flour Type 1 from Brazil, containing 12% carbohydrates, 7% proteins, 1% total fat, 5% dietary fiber, and whole wheat flour from Brazil, containing 12% carbohydrates, 8% proteins, 1% total fat, 24% dietary fiber, 1% Ca, 34% Fe. Gluten by MV Química (Barueri, SP, Brazil) containing 7.2% moisture, 0.57% ashes, 75.4% proteins, 161.00% water absorption, and 99.8% granulometry-mesh 80.

2.2. Microorganisms

2.2.1. Probiotics Strains

The *Bacillus licheniformis* LMG 12363 and *Saccharomyces boulardii* MUCL 43341 were obtained from Belgian Coordinated Collections of Microorganisms. The *Bacillus subtilis* LFB-FIOCRUZ 1267 obtained from the Culture Collection of *Bacillus* at the Fundação Oswaldo Cruz (Rio de Janeiro, RJ, Brazil) was used as a positive standard for detecting amylases, cellulases, and peptidases. The strains were cultivated in a Yeast Extract medium (5 g/L yeast extract, 5 g/L peptone, 20 g/L sucrose, 20 g/L KCl). The bacteria were cultivated at 37 °C for 24 h and yeast at 28 °C for 48 h. The strains were maintained in a medium culture agar slant tube at 4 °C for daily use and cryopreserved with glycerol 20% in a freezer at −18 °C. The Bacillus subtilis LFB-FIOCRUZ 1267 was used as a positive standard for detecting amylases, cellulases, and peptidases.

2.2.2. Isolation of Bacteria and Yeasts from Sourdough

Sourdoughs were produced based on the traditional protocol, according to Coda et al. [19] with modification. Two sourdoughs were produced: sourdough 1 (white wheat flour, WF) and sourdough 2 (whole wheat flour, WOF). In both types, 50 mL of sterile Milli-Q water was added to each result in a final dough yield (DY) (dough weight × 100/flour weight) of 200. The sourdoughs were propagated through daily back-slopping for ten days, mixing 50 g of the previous dough with 25 g of respective flours and 25 g of sterile Mili-Q water, maintaining a DY of 200. The doughs were incubated at room temperature (28 °C) for 4 days, then refrigerated at 4 °C until the 10th day. Samples were collected on the 4th and 10th day. Sourdough samples (1 g) were diluted in peptone water (9 mL) using a ten-fold dilution series. The diluted samples of lactic acid bacteria were spread-plated on an MRS medium with 0.01 g/L of nystatin and incubated anaerobically at 30 °C for 48 h, using shaker-incubator LAC−INA−800 (LACTEA, Cambuci, SP, Brazil). For total mesophilic aerobic microorganisms, the diluted samples were spread-plated on BHI, Yeast Extract medium, and Plate Count Agar (5.0 g/L enzymatic digest of casein/tryptone, 2.5 g/L yeast extract, 1.0 g/L glucose, and 20.0 g/L agar), and incubated at 30 °C for 48 h. For yeasts, the diluted samples were spread-plated on a Yeast Extract medium (according to Section 2.2.1) and incubated at 28 °C for 48–72 h. Colonies were picked randomly from plates containing 100 to 300 colonies, subcultured in the corresponding medium, and re-streaked onto the same medium with 2% agar to isolate colonies. The bacteria and yeasts were stored in agar slant tubes at 4 °C for further identification.

2.3. Microorganisms Identification

2.3.1. Matrix-Assisted Laser Desorption-Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF)

A single colony forming unit (CFU), isolated from a fresh culture, was spotted on Bruker's ground steel target plate (Bruker S.A.S., Wissembourg, France). Then, 1 µL of 70% formic acid was overlaid on each spot. After air drying at room temperature, 1 µL of the α-cyano-4 hydroxy-cinnamic acid matrix solution was added and air dried. Finally, the MALDI-TOF MS profiles were obtained using MALDI Microflex LT (Bruker Daltonics, Bremen, Germany) [20,21].

Identification results based on MALDI-TOF were accepted at the genus or species level, following Bruker's instructions. Only identifications with score > 2.00 were considered. Scores below 2.00 were selected for 16S rDNA analysis.

2.3.2. 16S rDNA Identification

The genomic DNA (gDNA) extraction was taken from the overgrown colonies using a commercial genomic DNA extraction kit. The integrity of the gDNA was verified through 1% agarose gel electrophoresis. The purity and concentration of the DNA samples were determined using the NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). The 16S rDNA identification was performed by the company AgregaBiotec (Porto Alegre, RS, Brazil). For the Polymerase Chain Reaction (PCR), 20 ng of gDNA, Taq DNA polymerase, 10X concentrated PCR buffer, $MgCl_2$, dNTPs, and primers 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) were used. The amplicon sequencing, based on the method of Sanger, was performed in both directions for the 16S rDNA amplicon obtained by PCR. To mark the template DNA, 2.5 pmol of primer (785F 5′-GGA TTA GAT ACCCTG GTA-3′ or 907R 5′-CCG TCA ATT CMT TTR AGT TT-3′) and 0.5 µL of bigDye reagent Terminator v3.1 Cycle Sequencing Kit Applied Biosystems (Thermo Fisher Scientific, Waltham, MA, USA) in a final volume of 10 µL were used. Sequencing data were collected using the Data Collection 3 program Applied Biosystems (Thermo Fisher Scientific, Waltham, MA, USA). The resulting Data Collection files (ab1; electropherograms) were converted into FASTA files (seq; text) by Sequence Analysis Software v. 6 Applied Biosystems (Thermo Fisher Scientific, Waltham, MA, USA) under standard parameters. Data analysis for each amplicon was conducted, and the final sequence was compared to 16S rDNA sequences of the genus of interest obtained from a database [22]. An outgroup was used for phylogenetic inference by Maximum Likelihood (ML). The outgroup was selected because it shared the same family as the analyzed genus, according to data from the Genome Taxonomy Database (GTDB, gtdb.ecogenomic.org/tree, accessed on 19 April 2023). Sequences were aligned with SINA 1.2.11 [23], and positions containing gaps were removed. Finally, a phylogenetic tree was calculated to build cladograms and identify microorganisms.

2.4. Hydrolases Detection

2.4.1. Detection of Amylase, Cellulase, and Peptidase Activity on Agar Plates

The selected microorganisms were subjected to the detection of extracellular hydrolases (amylase, cellulase, and peptidase). The tests were conducted on agar plates with the enzymatic extracts obtained from each microorganism after growth in its respective culture medium. For the screening of amylase, cellulase, and peptidase detection on agar plates, the microorganisms were cultured in their specific media for 24 h. Subsequently, the cultures were centrifuged at 5000 rpm for 20 min at 4 °C using centrifuge Sorvall ST 16R (Thermo Scientific, Waltham, MA, USA). The resulting supernatants were collected as enzymatic extracts for further analysis, following the method described by Junior et al. [24]. For amylase detection, the medium consisted of 2 g/L starch, 1 g/L yeast extract, and 8.5 g/L NaCl. For cellulase detection, the medium contained carboxymethylcellulose (CMC) at a concentration of 2 g/L, 1 g/L yeast extract, and 8.5 g/L NaCl. A gelatin medium was prepared with 10 g/L gelatin, 1 g/L yeast extract, and 8.5 g/L NaCl for peptidase detection.

All media used for enzymatic screening contained 2.0 g of agar. For the hydrolases assay, 70 µL of the enzymatic extract was added to a 5 mm well made in the agar plate. The plates were then incubated at 30 °C for 48 h to allow the enzymes to act. To visualize the hydrolysis halos caused by amylase and cellulase, a solution of Lugol 2% (containing 0.7% KI and 0.3% I_2) was used. A solution of Coomassie blue (0.25% w/v) in a mixture of methanol and acetic acid (5:1:4 $v/v/v$) was employed for peptidase activity. The plates were subsequently destained using a solution of 50% methanol and 10% acetic acid (v/v). All hydrolase activities were scored as grades: "grade -" when no visible halo was present, "grade +" when the visible halo was less than 2.0 cm, and "grade ++" when the halo zone was equal to or greater than 2.0 cm [25]. The experiments were performed in triplicate.

2.4.2. Quantitative Assay for Amylase, Cellulase, and Peptidase

Amylase activity was estimated using the DNS method [26] with minor modifications. Briefly, 500 µL enzymatic extract with 500 µL of DNS was boiled at 100 °C for 5 min. The quantity of enzyme was measured at 540 nm using UV–Vis Spectrophotometer SpectraMax i3x (Molecular Devices, San Jose, CA, USA), with a blank sample as a reference (enzymatic extract inactivated by boiled at 70 °C for 30 min). One unit (U) of enzyme activity was defined as the amount of amylase to produce 1 µmol of glucose per minute under the assay conditions. Amylase activity was performed in triplicate.

The cellulase activity was measured by reducing sugars liberated from CMC (2%) solubilized in 50 nM citrate buffer, pH 5.0 using pH meter AT–355 (Alfakit, Florianópolis, SC, Brazil). This mixture was incubated for 30 min at 50 °C. The reactions were stopped by adding DNS reagent and boiling for 5 min. The absorbance was read at 540 nm. The reducing sugars formed were quantified using glucose as standard. One unit (U) was defined as the amount of enzyme that releases 1 µmol of reducing sugar per minute. The experiments were performed in triplicate [24].

The quantification of peptidase activity was performed for gelatinase. The gelatinase assay was described by Mazotto et al. [27]. The supernatants of all assays were measured by absorbance at 660 nm to determine the protein concentration through the Lowry method using BSA (bovine serum albumin) as a standard [28]. The amount of enzyme necessary to increase the protein concentration in 1 µg was defined as one unit of activity (U) [29]. The experiments were performed in triplicate.

2.4.3. Analysis of Gluten and Wheat Flour Protein Hydrolysis by Peptidases

This study used a novel screening methodology to evaluate the capability of microorganisms to hydrolyze gluten and wheat flour proteins based on Vermelho et al. [25]. In each experiment, 70 µL of crude enzymatic extracts from microorganisms with peptidase activity, as described in Section 2.4.1, were applied to the wells of Petri dishes with medium (10 g/L gluten, 20 g/L agar) and medium (10 g/L white wheat flour, 20 g/L agar), respectively. The Petri dishes were then incubated at 30 °C for 48 h. Hydrolysis zones were detected using Coomassie blue staining and washed with a destaining solution (50% v/v Methanol, 10% v/v acetic acid). The gluten hydrolysis (glutenase) and the activity of peptidases in wheat flour protein substrate were categorized based on a grading system: "grade −" indicated the absence of a visible halo, "grade +" indicated visible proteolysis limited to smaller than 2.0 cm diameter halo, and "grade ++" indicated a proteolysis zone extending 2.0 cm diameter or more. The experiments were performed in triplicate.

2.5. Analysis of Leavening Effect of Yeasts Using White Wheat Flour

Yeast samples were selected based on the better enzymatic profile as described in Section 2.4.1. Five doughs were prepared using containers with the same shape and size. Each dough consisted of 15 g of white wheat flour, 18 g of filtered water, and 0.3 g of each sample with 10^9 CFU/mL, and no inoculated dough (control) was used as a reference. The doughs were incubated at 35 °C for 3 h. At the end of the period, the heights of each

sample were measured [30,31]. The sample with the best performance after fermentation was selected for analyses as described in Sections 2.4.2, 2.4.3 and 2.6.

2.6. Electrophoretic Analyses

These experiments were carried out with the microorganisms that showed the best hydrolysis screening results and leavening ability.

Gluten Zymography and Enzymography

Proteolytic activities were characterized using 10% SDS-PAGE copolymerized with 10% of the supernatant after gluten extraction with the buffer Tris-HCl pH 8.8 1.5 M (*w*/*v*). The gel was loaded with 40 µL of enzyme extract concentrated on a 10,000 kDa membrane Amicon (Sigma-Aldrich, Burlington, MA, USA) per slot and subjected to electrophoresis at a constant voltage of 170 V, 400 mA at 4 °C for 2 h, using Mini PROTEAN® Tetra Cell (Bio-Rad, Hercules, CA, USA). After that, the gel was washed twice with Triton X100 for 15 min each. Finally, the gel was stained with Coomassie blue R250 solution for 24 h and destained with methanol and acetic acid solutions [27]. Enzymography was applied to detect gluten hydrolysis. The crude enzyme extract supernatants of microorganisms were mixed with 60 µg of gluten powder diluted in phosphate buffer (0.06 M $Na_2HPO_4 \cdot 7H_2O$/0.04 M KH_2PO_4, pH 7.2). The reaction mixtures were incubated for 10 min, 20 min, 30 min, 1 h, 2 h, and 4 h at 37 °C. The reactions were stopped by adding sample protein buffer (250 mM Tris-HCL, 5% *v*/*v* 2-mercaptoethanol, 10% *w*/*v* SDS, 30% *w*/*v* glycerol, 0.02% *w*/*v* bromophenol blue, pH 6.8), and boiled at 100 °C for 5 min. A volume of 40 µL of the samples was added per slot, and following electrophoresis, 10% SDS-PAGE gel at a constant voltage of 170 V, 400 mA at room temperature for 2 h, staining with Coomassie brilliant blue for 12 h, and then destaining in washing buffer (50% *v*/*v* Methanol, 10% *v*/*v* acetic acid) for 1 h [27].

2.7. Statistical Analysis

All statistical analyses were performed using R (v4.2) [32]. A Shapiro–Wilk test was performed to check data normality, after that Kruskal–Wallis and Dunn's tests were used to evaluate the statistical significance of the data. Post hoc analyses were performed using the R package postHoc [33]. Statistical significance was considered when $p < 0.05$.

3. Results and Discussion

3.1. Microorganisms Identification

Initially, microorganisms were isolated from different wheat flour samples to screen strains producing enzymes relevant to the baking industry. The identification of the isolated microorganisms was performed through MALDI-TOF and 16S rDNA, where the selection of the strains of interest was performed through qualitative enzymatic methods. Table 1 shows the microorganisms identified in the flours (MALDI-TOF) and species similarity (16S rDNA). In total, 14 strains were identified by MALDI-TOF and 4 by 16S rDNA.

The isolation of genera belonging to the phylum Pseudomonadota (*K. cowanii* (S), and *P. agglomerans* (S)) in fresh sourdough aligns with previous studies that obtained similar results in sourdough cultures refreshed up to four days [34]. This phylum is commonly associated with wheat flour. In mature sourdough, the dominant group is the lactic acid bacteria (LAB) independent of using a LAB starter [35].

The decrease in pH during sourdough fermentation, with levels below 5, can inhibit the growth of Enterobacteriaceae [36]. However, a pH of 4 is a safe mark to determine mature sourdough. Nonetheless, genera such as *Pantoea*, *Pseudomonas*, and *Kosakonia* may persist if the fermentation temperature reaches 30 ± 1 °C, as observed in a study conducted in Brazil [37].

Table 1. Microorganisms' identification by MALDI TOF and 16S rDNA.

Identification Method	Microorganism	Sourdough
MALDI-TOF	*Bacillus cereus* (S)	WF
MALDI-TOF	*Candida guilliermondii* (S)	WF
MALDI-TOF	*Enterococcus faecium* (S1)	WF
MALDI-TOF	*Enterococcus faecium* (S2)	WF
MALDI-TOF	*Kazachstania unispora* (S)	WOF
MALDI-TOF	*Kosakonia cowanii* (S)	WF
MALDI-TOF	*Lactobacillus plantarum* (S)	WF
MALDI-TOF	*Limosilactobacillus fermentum* (S)	WF
MALDI-TOF	*Pantoea agglomerans* (S)	WOF
MALDI-TOF	*Pediococcus acidilactici* (S)	WF
MALDI-TOF	*Pediococcus pentosaceus* (S)	WF
MALDI-TOF	*Rhodotorula mucilaginosa* (S)	WF
MALDI-TOF	*Saccharomyces cerevisiae* (S1)	WF
MALDI-TOF	*Saccharomyces cerevisiae* (S2)	WF
16S rDNA	*Bacillus* sp. (S1)	WOF
16S rDNA	*Bacillus* sp. (S2)	WF
16S rDNA	*Lactiplantibacillus* sp. (S)	WF
16S rDNA	*Paraburkholderia* sp. (S)	WOF

WF, white wheat flour; WOF, whole wheat flour. (S), microorganisms isolated from sourdough. Numbers "1" and "2" refer to the same genus/specie isolated with different characteristics.

Related LAB species, namely, *L. plantarum* (S), *P. pentosaceus* (S), and *P. acidilactici* (S), were isolated after ten days of consecutive back-slopping. Among these, *L. plantarum* is the most frequently found hetero-fermentative species in the sourdough environment, as mentioned in 142 out of 312 research articles [38]. Although *Lactobacillus* is generally more abundant in sourdough, *Pediococcus* is found in smaller quantities or rarely occurs [39,40].

Regarding wild yeasts, the ratio of LAB to yeast in sourdough typically ranges from 100:1 to 10:1 [41]. *K. unispora* (S) was isolated from WOF, and *R. mucilaginosa* (S) from WF, both are reported as rarely in sourdough by De Vuyst et al. [42]. *S. cerevisiae* is the most predominant yeast in sourdough microbiota [37], and two strains, *S. cerevisiae* (S1) and *S. cerevisiae* (S2), were isolated from WF.

Bacillus presence in wheat flour is usual [43]. A study showed the importance of analyzing the role of the *Bacillus* genus in sourdough fermentation. They were found throughout the entire sourdough fermentation process for 14 and 39 days in the Portuguese broa sourdough production [44]. Moreover, *Bacillus brevis*, *Bacillus cereus*, *Bacillus circulans*, *Bacillus laterosporus*, *Bacillus licheniformis*, *Bacillus macerans*, *Bacillus megaterium*, *Bacillus mycoides*, *Bacillus polymyxa*, *Bacillus pumilus*, *Bacillus stearothermophilus*, and *Bacillus subtilis* were isolated from Portuguese sourdough for bread production with maize and rye [45]. *Bacillus* species isolated from Adhirasam, a rice-fermented doughnut from South India, were used as starter cultures and produced Adhirasam with superior quality [46]. *Bacillus* species are found in several fermented foods. They are recognized for producing hydrolases, extracellular polysaccharides, and lipopeptides with antimicrobial activity that could be advantageous for their use as starters in fermented foods [47]. *Paraburkholderia* is not related to wheat sourdough, but Brazil is known as a diversity center of this microorganism [48]. It is also associated with a fermented cereal non-alcoholic beverage from Nigeria [49].

3.2. Qualitative and Quantitative Hydrolases Analysis

After identifying the microorganisms isolated from sourdough, these microorganisms and the probiotic strains were used in qualitative enzyme assays to evaluate the production of amylase, cellulase, peptidase, wheat flour peptidase, and gluten peptidase on agar plates. Table 2 summarizes all results for the enzymes obtained with the diffusion method on agar plates. As a result, four strains stood out in the simultaneous production of the hydrolases for all evaluated substrates: *L. fermentum* (S), *B. licheniformis* LMG 12363 (P), *P. Pentosaceus*

(S), and *S. cerevisiae* (S2). The *B. subtilis* from the FIOCRUZ collection was used as the positive control.

Table 2. Hydrolysis screening results for isolated microorganisms (S) and acquired probiotics (P).

Microorganisms	Amylase	Cellulase	Peptidase	Wheat Peptidase	Glutenase
B. licheniformis LMG 12363 (P)	+	+	+	+	++
E. faecium (S1)	-	++	-	-	-
E. faecium (S2)	-	-	+	+	+
K. unispora (S)	+	-	+	+	+
L. plantarum (S)	-	-	+	+	+
L. fermentum (S)	++	+	++	++	+
P. acidilactici (S)	-	++	+	+	+
P. pentosaceus (S)	+	+	+	+	+
S. boulardii MUCL 43341 (P)	+	-	+	+	+
S. cerevisiae (S1)	+	-	+	+	+
S. cerevisiae (S2)	++	+	++	++	+

alues: - no visible halo; + visible halo smaller than 2.0 cm diameter; ++ visible halo of 2.0 cm diameter or larger. (P), Probiotic strains; (S), microorganisms isolated from sourdough. (S1) and (S2) are two genera or species with different enzymatic profiles.

The enzymatic profile was heterogeneous, but it is interesting to note that some bacteria can degrade gluten, such as the probiotics *B. liqueniformis* LMG 12363 (P), *L. fermentum* (S), and the *S. boulardii* MUCL 43341 (P), as well the *E. faecium* (S2), *L. plantarum* (S), *P. acidilactici* (S), *P. pentosaceus* (S), *K. unispora* (S), and two *S. cerevisiae* isolates from sourdough. *L. fermentum* (S) and *S. cerevisiae* (S2) showed similarity in the enzymatic profile, with halos of hydrolysis for amylase, peptidase, and peptidase for wheat flour and gluten.

For bread technology, the amount of amylase in the dough contributes to liberating fermentable sugars for fermentation, which can interfere with the velocity of fermentation. Additionally, the enzyme acts in starch retrogradation properties, which can interfere with bread characteristics and modify the color of crumb and crust due to the Maillard reactions [50]. The strains *B. licheniformis* LMG 12363 (P), *L. fermentum* (S), *P. pentosaceus* (S), and *S. cerevisiae* (S2), showed activity for all analyzed enzymes and then were selected for quantitative enzymatic analysis and gluten electrophoresis. Although amylase activity in fermentation is essential, the balance of the amount is crucial to support the final starch structure to maintain the bread quality [13,51]. In the screening for amylase, all yeasts presented hydrolysis halo; *S. cerevisiae* (S2) showed the most expressive halo (++). The bacteria *B. licheniformis* LMG 12363 (P), *L. fermentum* (S), and *P. pentosaceus* (S) presented a hydrolysis halo, with emphasis on *L. fermentum* (S) (++). For amylolytic activity, *B. licheniformis* LMG 12363 (P) (0.413 µmol/mL $\pm$ 0.016) presented the lowest activity in the quantitative method. *S. cerevisiae* (S2) (3.98 µmol/mL $\pm$ 0.186) showed the highest activity, followed by *P. pentosaceus* (S) (3.698 µmol/mL $\pm$ 0.074) and *L. fermentum* (S) (2.21 µmol/mL $\pm$ 0.017) (Figure 1).

Studies reported the amylolytic activity of *B. licheniformis*. The strain *B. subtilis* WB 600 was genetically modified to overexpress the maltogenic amylase gene from *B. licheniformis*. As a result, the strain promoted better bread volume and elasticity, collaborating to increase the product's shelf life [52]. Another study reported *B. licheniformis* YB-1234 isolated from fermented soybeans capable of producing thermostable α-amylase [53]. In this way, our result for *B. licheniformis* LMG 12363 demonstrates the potential of the strain. However, further in-depth studies are needed for its use in the production of bakery products.

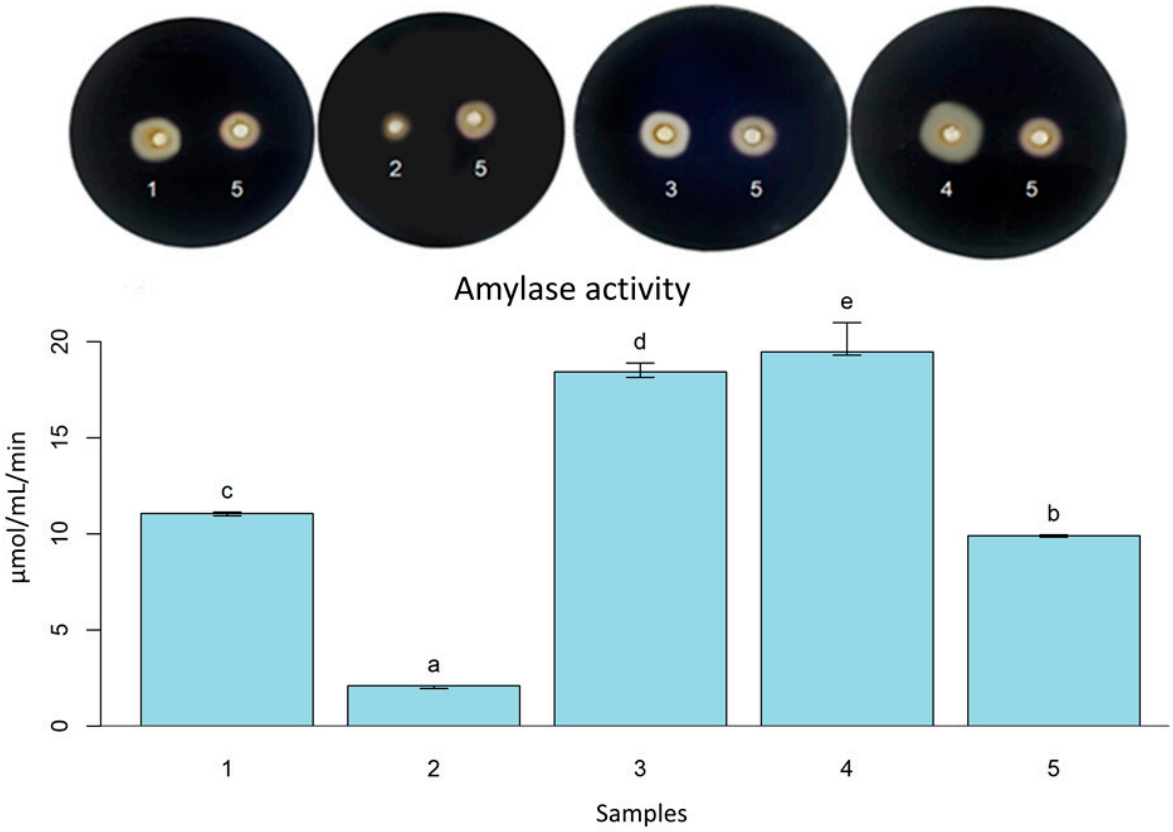

Figure 1. Amylase activity with Petri dishes screening of *L. fermentum* (S) (1); *B. licheniformis* LMG 12363 (P) (2); *P. pentosaceus* (S) (3); *S. cerevisiae* (S2) (4); *B. subtilis* LFB-FIOCRUZ 1267 (5). Different letters indicate statistically significant ($p < 0.05$) differences between the samples.

The strains *L. plantarum* and *L. fermentum* present amylolytic activity below pH 4.0 [54,55]; this feature may provide the availability of fermentable sugar during the entire fermentation process, which can be seen as a fermentation advantage since the amylase from wheat is deactivated in low pH [13]. Our result for the amylase activity of *L. fermentum* (S) was similar to the strain *L. fermentum* EN17-2, which exhibited α-amylase activity of 2.00 U/mL in the pH range of 3.5 to 5.5 [55].

S. cerevisiae is not recognized as a great amylase producer, except for *S. cerevisiae* var. *diastaticus*, which produces glucoamylase [56]. However, *S. cerevisiae* produces α-glucosidase that catalyzes the liberation of α-glucose from nonreducing ends of α-glucosides or from complex polymers with α-(1-4) bonds, such as malto-oligosaccharides, soluble starch, amylose, and glycogen [57]. A study showed that 2% potato peel increased the amylase activity of *S. cerevisiae*, and 4% potato peel improved cellulase activity, and the bread properties, such as retard staling and sensory quality [58]. Olasupo et al. [59] isolated and characterized an amylolytic strain of *S. cerevisiae* from yam tuber for the brewing industry in Nigeria. Additionally, *S. cerevisiae* NJJUM 13 isolated from Mangrove Environ showed amylase hydrolysis halo (2.2 cm) [60], similar to our result for *S. cerevisiae* (S2) with 2.1 cm. *P. pentosaceus* PKL-17 strain isolated from traditional pickles showed amylase, cellulase, and peptidase activity [61]. This result agrees with our work demonstrating high amylase activity by *P. pentosaceus* (S). However, this activity is not ubiquitous, as an α-amylase inhibitory capacity was described for a strain of *P. pentosaceus*. The authors suggest that this can be an interesting property. It could be used as an anti-diabetic probiotic [62].

The cellulase role in bread technology has been described to promote iron bioaccessibility [63]. This enzyme can act positively in gluten network promoting the anti-staling process, elasticity, volume, and softness of bread [64].

Enterococcus faecium (S1) and *P.* acidilactici (S) showed a significant hydrolysis halo in the cellulase screening. Only *S. cerevisiae* (S2) among yeasts exhibited cellulose hydrolysis (Table 2). In the cellulase activity, *P. pentosaceus* (S) showed the highest value (0.493 U/mL ± 0.058), and the *B. lichehiformis* LMG 12363 (P) (0.307 U/mL ± 0.020) was the smallest value close the *L. fermentum* (S) (0.363 U/mL ± 0.060) and *S. cerevisiae* (S2) (0.375 U/mL ± 0.060) (Figure 2).

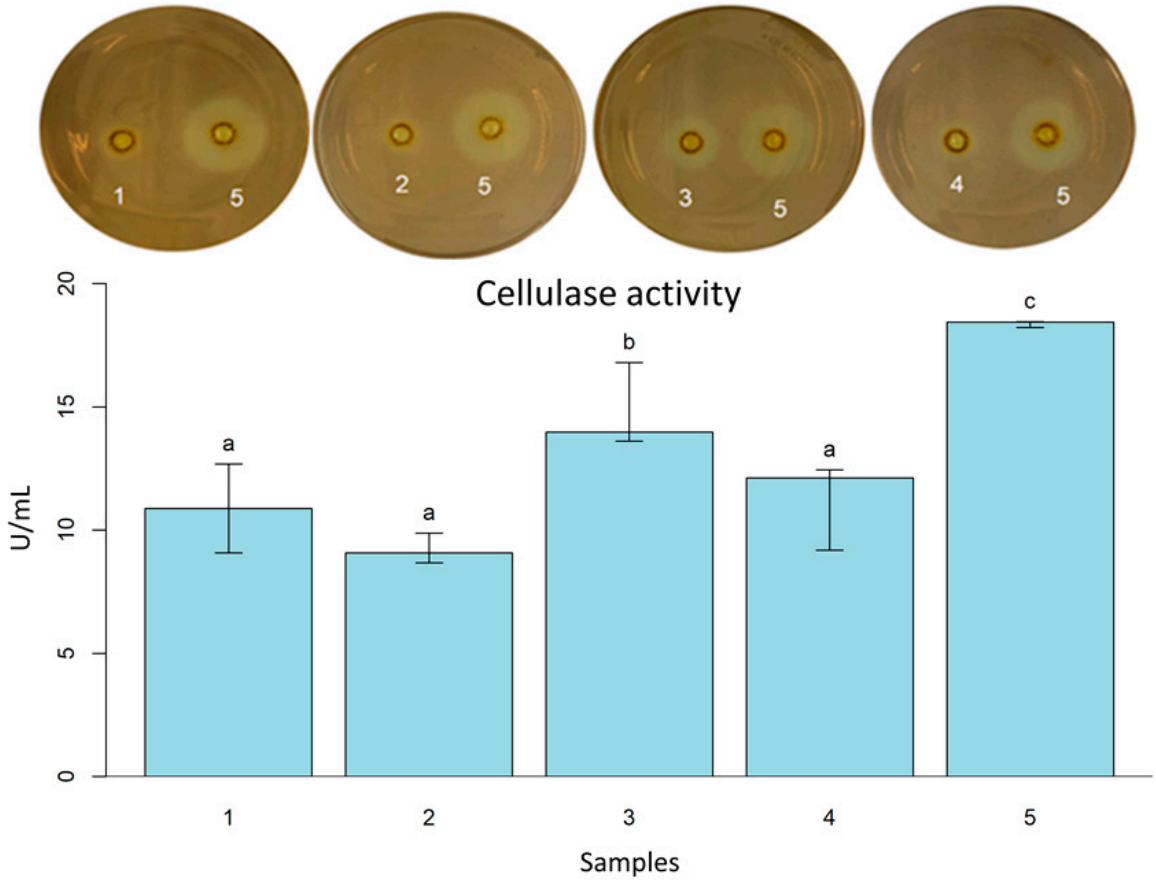

Figure 2. Cellulase activity with Petri dishes screening of *L. fermentum* (S) (1); *B. licheniformis* LMG 12363 (P) (2); *P. pentosaceus* (S) (3); *S. cerevisiae* (S2) (4); *B. subtilis* LFB-FIOCRUZ 1267(5). Different letters indicate statistically significant ($p < 0.05$) differences between the samples.

Cellulase has been associated with improvements in bread, particularly those with a high dietary fiber content [64,65]. It has been described that the presence of peptidases and cellulases of *B. licheniformis* isolated from camel feces can reduce allergenic compounds and improve the nutrition properties of soybean meal, which is a source of protein for animal feeds [66]. *B. licheniformis* MVS1 and *Bacillus* sp. MVS3 isolated from hot springs showed variation in cellulase activity with changes in nitrogen sources, carbon substrates, pH, temperature, and incubation time. According to the authors, *B. licheniformis* MVS1 exhibited the highest cellulase activity (0.120 ± 0.012 IU/mL) when wheat straw was the carbon source. However, when cultivated in a culture medium with yeast extract and carboxymethylcellulose, the cellulase activity of *B. licheniformis* MVS1 was reduced to 0.041 ± 0.003 IU/mL [67].

All analyzed strains have a performance that suggests the potential for future applications in fiber-rich bakery products, such as products made with whole wheat flour or incorporating grains into the dough. However, more investigations are needed to explore using active cellulase inoculants in sourdough preparations.

The peptidase activity in bread fermentation has significant importance since it can liberate peptides with antimicrobial properties [68], flavor's precursors, bioactive pep-

tides, and anti-hypertensive tripeptides [13,69], as well as improve volume, texture, and reduce staling ratio when it is an appropriate amount [70]. In addition, it was observed that peptidases from *Lactobacillus acidophilus* 5e2 and *Aspergillus ninger* could reduce gliadins and coeliac-toxic peptides during the bread-making process [71]. *P. acidilactici* XZ31 with *S. cerevisiae* JM4 degraded gluten peptides and reduced gluten immunogenicity [9]. *L. fermentum* 3872 produced bacteriolysin BLF3872 with a lysozyme-like domain and peptidase M23 domain [72].

As shown in Table 2, some strains presented positive results for peptidases, such as *B. licheniformis* LMG 12363 (P), *L. fermentum* (S), *E. faecium* (S2), *L. plantarum* (S), *P. acidilactici* (S), *P. pentosaceus* (S), and all analyzed yeasts. Additionally, those strains presented a capacity for wheat flour and gluten hydrolysis. These findings are highly significant due to the interest in understanding the impact of peptidases on baking technology. *L. fermentum* (S) exhibited the highest gelatinase activity (205.64 U/mL ± 5.888). *B. licheniformis* LMG 12363 (P) (20.89 U/mL ± 6.841) was the second followed by *S. cerevisiae* (S2) (12.051 ± 4.778), and *P. pentosaceus* (S) (6.02 U/mL ± 5.605) (Figure 3).

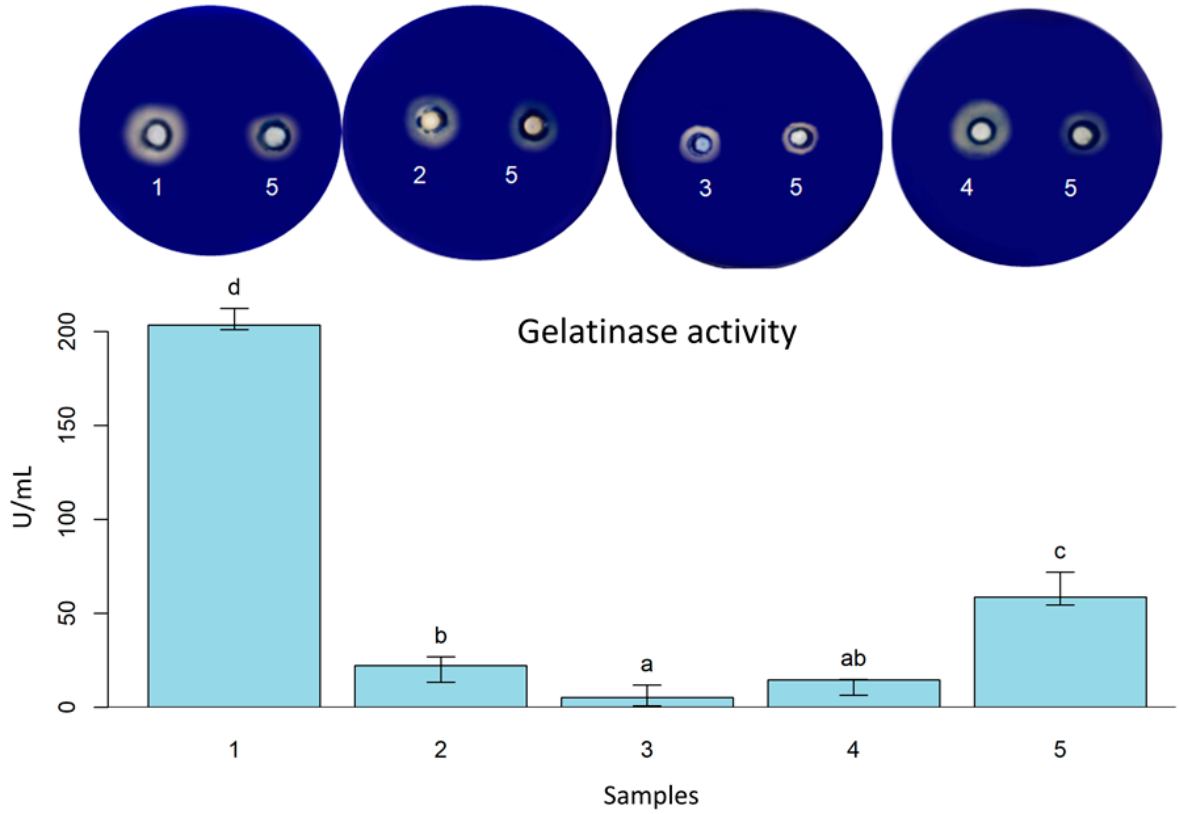

Figure 3. Gelatinase activity with Petri dishes screening of *L. fermentum* (S) (1); *B. licheniformis* LMG 12363 (P) (2); *P. pentosaceus* (S) (3); *S. cerevisiae* (S2) (4); *B. subtilis* LFB-FIOCRUZ 1267 (5). Different letters indicate statistically significant ($p < 0.05$) differences between the samples.

Bacillus and *Aspergillus* are recognized as primary peptidase producers [73]. A thermostable serine protease from *B. licheniformis* (LMG7561) reduced staling, promoted softness, and the effect was additive to known anti-staling agents (such as amylases) [74]. In another study, a serine protease with properties to be used in the food industry was purified and characterized from *B. licheniformis* KB111 [75]. The *Bacillus* genus is involved in bread spoilage [76]. However, some *Bacillus* species are recognized as Generally Recognized as Safe (GRAS), and probiotics can improve nutritionally and technologically gluten products with their cells or enzymes [47]. In our study, for instance, the strain *B. licheniformis* LMG 12363 (P) had the highest gluten hydrolysis halo, followed by *P. pentosaceus* (S) and *S. cere-*

visiae (S2). In terms of wheat flour hydrolysis, *L. fermentum* (S) stood out for its hydrolysis halo performance, followed by *S. cerevisiae* (S2) (Figure 4).

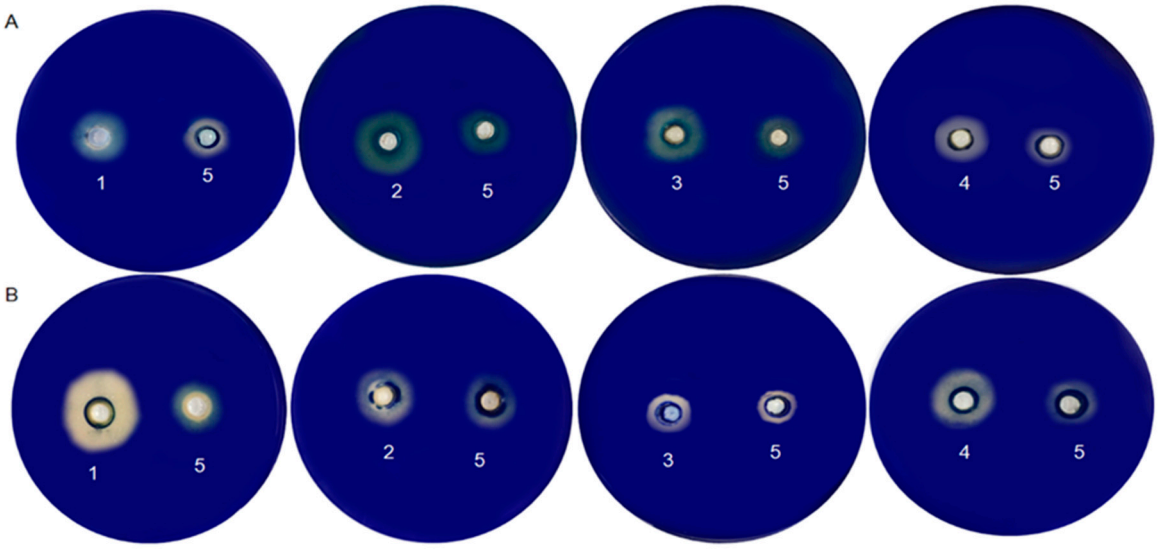

Figure 4. Gluten protein hydrolysis screening (**A**) and wheat flour protein hydrolysis screening (**B**): *L. fermentum* (S) (1); *Bacillus licheniformis* LMG 12363 (P) (2); *P. pentosaceus* (S) (3); *S. cerevisiae* (S2) (4); *B. subtilis* LFB-FIOCRUZ 1267 (5).

Peptidase can break down both soluble and insoluble proteins in wheat [11,77,78]. When comparing our results of wheat flour and gluten hydrolysis, we observed that *L. fermentum* (S), *B. licheniformis* LMG 12363, *P. pentosaceus* (S), and *S. cerevisiae* (S2) presented hydrolysis halo for both types. The combination of *S. cerevisiae* (S2) and selected probiotic bacteria could enhance the fermentation and enzymatic activity of bakery products, resulting in novel characteristics. Further research is required to evaluate the optimal conditions and benefits of using these strains.

3.3. Leavening Effect of Yeasts

The objective of leavening dough is to generate and retain carbon dioxide (CO_2) within the dough structure. CO_2 production is linked to the yeast's ability to transform available fermentable carbohydrates in the dough through ethanolic fermentation. Certain bacteria, such as obligatory heterofermentative LAB (OHLAB), also contribute to CO_2 production. Combining *S. cerevisiae* with OHLAB from sourdough significantly improved the leavening capacity of dough [30].

The primary source of carbohydrates in wheat is starch, which accounts for 70–75% of wheat flour. Starch undergoes hydrolysis by amylases, resulting in the production of fermentable sugars. Additionally, wheat flour contains slight amounts of readily fermentable sugars such as glucose, sucrose, maltose, fructose, maltotriose, and raffinose [79]. The hydrolysis of maltose by *S. cerevisiae* is responsible for the speed of fermentation, and this capability may be attributed to its adaptation during its long history in bread-making [80].

In this study, more than one strain of *S. cerevisiae* produced hydrolases (amylases and cellulases) which can liberate carbohydrates and, subsequently, through ethanolic fermentation, produce CO_2. A method for evaluating fermentative capacity was used as a tie-breaker test. In this case, the strains were inoculated in wheat flour and then placed for fermentation under controlled conditions. Figure 5A shows the fermentative performance of the evaluated strains. The height data were tested for normality using the Shapiro–Wilk test. Non-parametric tests (Kruskal–Wallis and Dunn's test) were used to compare the height of the samples ($\alpha = 0.05$). Sample 3 was the only sample that presented height with a statistical difference from the control (Figure 5B). Thus, it was possible to choose strain *S. cerevisiae* (S2) due to its superior fermentation volume.

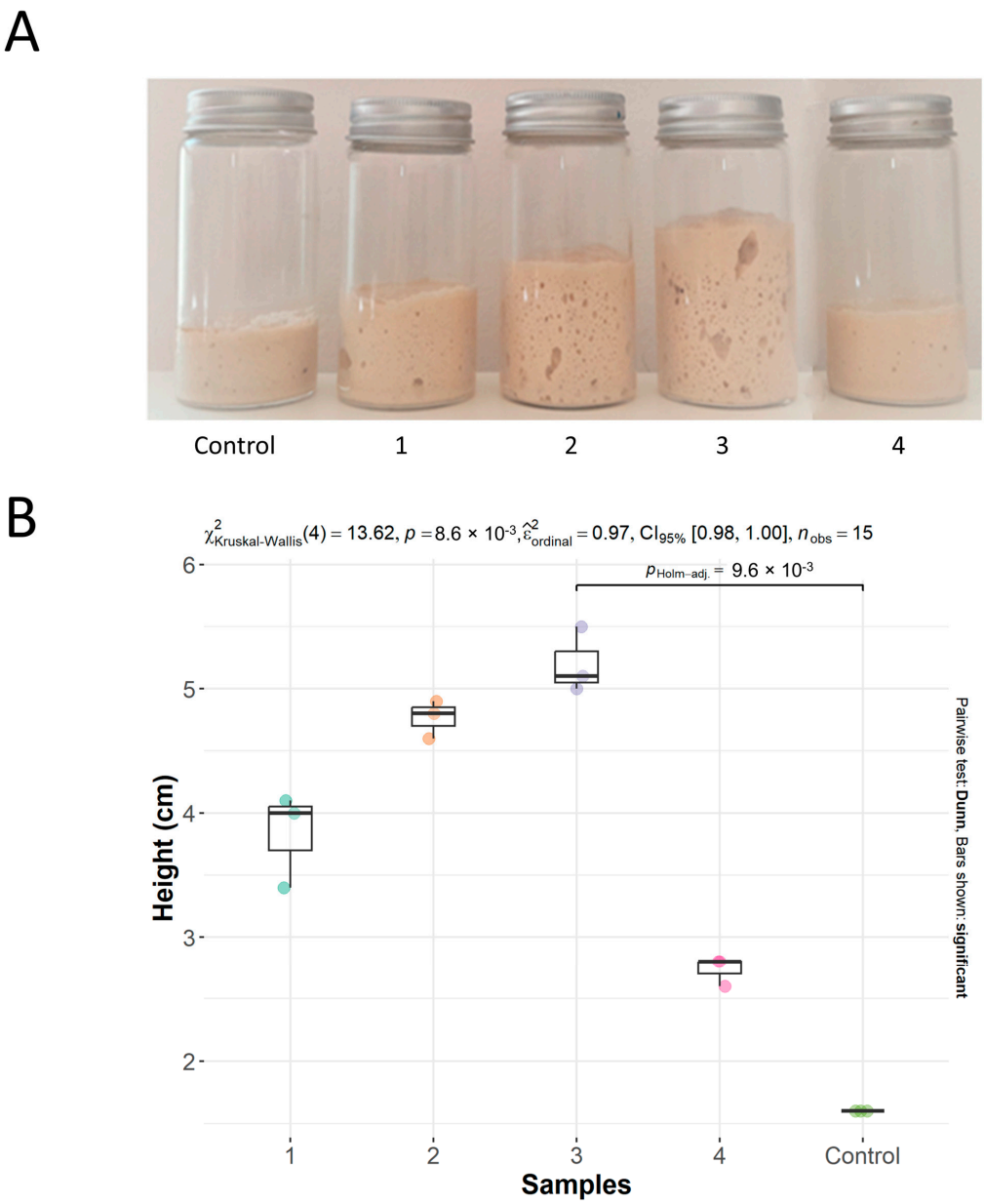

Figure 5. (**A**) Dough without inoculum (Control); dough with *S. boulardii* MUCL 43341 (P) (1); dough with *S. cerevisiae* (S1) (2); dough with *S. cerevisiae* (S2) (3); dough with *K. unispora* (S) (4). (**B**) Boxplot of height (cm) of levain fermentation in tubes. The line represents the only sample (3) that presented height with a statistically significant difference compared to the control ($p < 0.05$).

K. unispora has been studied for its consistent presence in sourdough and fermented foods, making it a potentially safe option for the food industry. A recent study showed an adequate leavening capacity for this yeast [81]. However, when compared to commercial *S. cerevisiae*, its performance was inferior. Our findings supported this finding, as the strain *K. unispora* (S) did not surpass the leavening ability of the *S. cerevisiae* samples. The probiotic sample *S. boulardii* MUCL 43341 (P) presented low performance. A study carried out with *S. boulardii* application to sourdough found that the bread had a distinct flavor and texture with good acceptance [82]. However, studies of *S. boulardii* performance in dough fermentation must be more assessed. Regarding the result for the two samples of *S. cerevisiae*, the highlight of the *S. cerevisiae* (S2) strain suggests the influence of its superior enzymatic profile for amylase and cellulase activity previously analyzed (Table 2).

3.4. Enzymography and Zymography

Enzymography and zymography are techniques for the separation and visualization of enzymes that can act in the degradation of a substrate, so they can be applied to the study of gluten and its degradation [83,84].

This study investigated the enzymatic activity of the selected microorganisms' crude extracts concerning gluten biodegradation. The enzymography technique was adopted to detect the enzymatic action on gluten at different incubation times (Figure 6). Gluten proteins are wheat storage proteins with hundreds of components that differ in solubility, structure, molecular weight, and amino acid composition. The enzymatic extracts of all microorganisms showed activity. Figure 6A shows the beginning of the experiment at time 0, where it is possible to observe no hydrolysis and consequent bands because the action of the microorganism's peptidases did not start effectively. Gluten is a macromolecule that must be hydrolyzed to enter the acrylamide gel. The biodegradation begins slowly, and in 10 min, it is possible to observe subtle bands starting to show (Figure 6B), at 30 min (Figure 6C) and at 2 h (Figure 6D). All extracts showed activity with bands around 30,000 MW, 45,000 MW, and 50,000 MW.

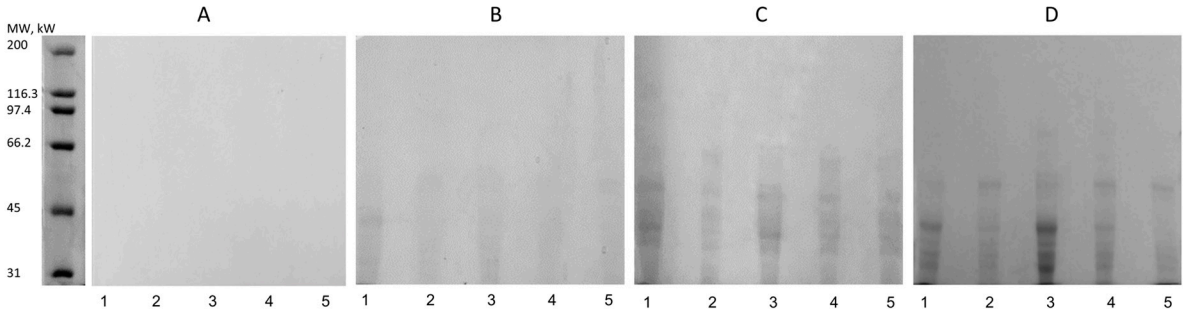

Figure 6. Enzymography of gluten hydrolysis over time (0–2 h) by extracellular enzymes of 1: *L. fermentum* (S); 2: *B. licheniformis* LMG 12363 (P); 3: *P. pentosaceus* (S); 4: *S. cerevisiae* (S2); and 5: *B. subtilis* LFB-FIOCRUZ 1267 used as control. The reaction mixtures containing culture supernatant and gluten solution were incubated for 0 min (**A**), 10 min (**B**), 30 min (**C**), and 2 h (**D**).

The diversity of MW ranges shows that the byproducts of gluten biodegradation can present very diverse fragments. Figure 6D shows the result of degradation after 2 h of incubation. It can be seen that Lane 3 showed bands and possibly up to ~66.2 kDa, while the other lanes reached a maximum of ~45 kDa. del Amo-Maestro et al. [85] used SDS-PAGE analysis to investigate the digestion of gliadin by neprosin. They observed that neprosin efficiently degraded gliadin at concentrations below ~5 µM, generating fragments below the control bands. Thus, the fragments shown in Figure 7 suggest gluten degradation. However, making any statement about the specificity of enzyme action is impossible.

After verifying the enzymatic activity of gluten by enzymography, we employed the zymography technique to evaluate the MW distribution of enzymes with activity on the fraction of gluten solubilized in the buffer Tris-HCl pH 8.8 1.5 M (*w*/*v*). Thus, it is possible to visualize the active enzymes in an electrophoresis gel directly. Figure 7 shows the zymography of the four extracts evaluated where it is possible to observe the presence of different patterns of clear bands in the gels, indicating a variety of enzymes with activity on gluten.

These bands demonstrate enzymatic activity in the degradation of gluten which is a complex protein. It is possible to notice that Lane 1 stands out in the diversity of enzymes with action on gluten, then Lanes 3–4, and lastly, Lane 2, which showed a smaller band. Thus, a more pronounced enzymatic activity in the extract *L. fermentum* (S) is suggested. In contrast, others showed smaller or less evident bands. Thus, the enzyme extracts show enzymes in the ~45–200 kDa range.

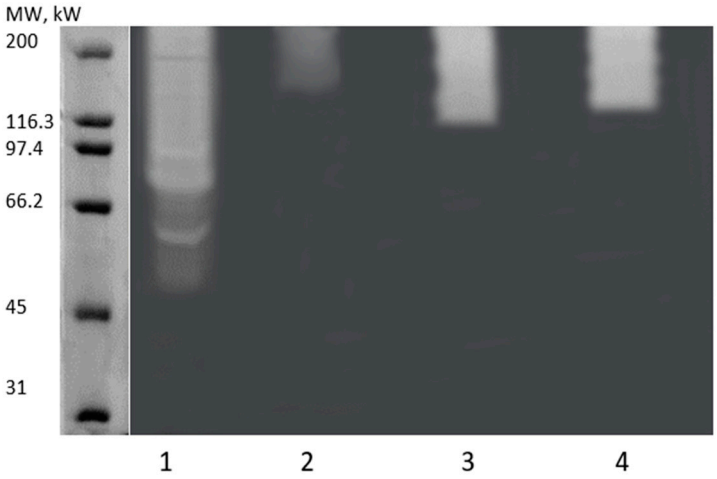

Figure 7. Zymogram analysis of extracellular enzymes from 1: *L. fermentum* (S); 2: *B. licheniformis* LMG 12363 (P); 3: *P. pentosaceus* (S); and 4: *S. cerevisiae* (S2). The molecular standard used is shown on the left.

Some peptidases can degrade gluten and have a MW range ranging from 20 to 35 kDa [86]. In the work by Ciurko et al. [87], the authors used zymography to determine the molecular weight of the proteolytic enzymes in different microbial extracts. As a result, they observed the presence of peptidases with high molecular weights, specifically with relative molecular masses of 100, 70, and 55 kDa. Another work demonstrated that *Bacillus polymyxa* could produce extracellular peptidase with molecular masses of 20, 35, 50, and 210 kDa [88]. Liu et al. [89] used a similar technique where they employed gliadin zymogram to analyze secreted peptidases from *Burkholderia gladioli*, *Burkholderia cepacia*, *Dyella japonica*, *Dyella yeojuensis*, *Pseudomonas aeruginosa*, and *Serratia marcescens*. The authors' zymogram shows the occurrence of peptidases in the range of 48–180 kDa, where they isolated a serine peptidase with a molecular mass of ~51.4 kDa.

This work and other gluten-related studies have reported protein clustering in the 180–200 kDa region. Wei et al. [90] identified a proteolytic enzyme (elastase) with a molecular weight of 53 kDa that was located in the ~200 kDa band. The authors claimed that protein complex formation cannot be excluded in native PAGE. Lu et al. [91] used gliadin zymography to characterize the molecular weight of gliadin-degrading enzymes from *B. cereus* strains. As a result, they observed active enzyme bands in the high molecular weight region (>170 kDa) and the ~55–72 kDa region. The authors claimed that the band exists in the high region due to dimeric forms of the low molecular weight enzymes [91].

The analysis of enzyme activity through the techniques of zymography and enzymography is of great importance since they allow a direct, visual analysis with information about MW and enzyme activity on gluten. This information is fundamental for understanding the enzymatic diversity of microorganisms involved in fermentation, and helps in the selection of promising strains for the baking industry. However, it is important to highlight that these results do not precisely characterize the involved enzymes.

Future studies should be conducted to purify and identify these enzymes, and investigate their biochemical properties and potential applications in the baking industry. These findings provide a promising basis to explore the enzymes produced by isolated microorganisms as tools to improve the quality of gluten and, consequently, baked goods.

4. Conclusions

Combining and balancing microorganisms, with enzymatic versatility, can offer advantages in reducing allergenic molecules in wheat flour while preserving the properties of bakery products. Hence, enrichment of the enzyme profile of fermented dough can improve the nutritional value and quality of the final product. Screening for gluten and wheat flour hydrolysis is the first step in selecting promising strains to reduce wheat-allergenic

molecules. The method we used proved to be simple and efficient in screening glutenase-producing microorganisms. This study identified several microorganisms from sourdough, including *Bacillus* strains. Characterizing these strains could mean more starters for use in the food industry. The addition of probiotic bacteria could represent an innovation in sourdough, improving and evolving its beneficial properties. The results of this work contributed to identify activities of hydrolases from sourdough microorganisms, which may help in the development of new applications in this type of fermentation. Furthermore, to the best of our knowledge, it was the first time that amylase, cellulase, and peptidase activities were evaluated from sourdough microorganisms.

Author Contributions: Conceptualization, experiments, and writing, I.T.A.; writing, formal analysis, and original draft preparation, F.R.P.M.; performing enzyme experiments, discussing, and integrating the data, V.S.C.; performing substrate characterization, and analyzing and discussing data; E.P.d.S.D.; conceptualization, writing, review, editing, project administration, and funding acquisition, A.B.V. All authors have read and agreed to the published version of the manuscript.

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